Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 145–150, January 1999
Cell Biology

Proapoptotic activity of *Caenorhabditis elegans* CED-4 protein in *Drosophila*: Implicated mechanisms for caspase activation

(programmed cell death)

HIROTAKA KANUKA*, SHIN HISAHARA*†, KAZUNOBU SAWAMOTO*‡, SHIN-ICHI SHOJI†, HIDEYUKI OKANO*‡§, AND MASAYUKI MIURA*‡§¶

*Department of Neuroanatomy, Biomedical Research Center, Osaka University Medical School, 2–2 Yamadaoka, Suita, Osaka 565-0871, Japan; †Department of Neurology, Institute of Clinical Medical Sciences, University of Tsukuba, Tsukuba, 1-1-1 Tennoudai, Ibaraki 305-0006, Japan; and ‡Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, 2-6-15 Shibakoen, Minato-ku, Tokyo 105-0011, Japan

Edited by Suzanne Cory, the Royal Melbourne Hospital, Melbourne, Australia, and approved November 5, 1998 (received for review March 11, 1998)

ABSTRACT **CED-4 protein plays an important role in the induction of programmed cell death in *Caenorhabditis elegans* through the activation of caspases. However, the precise mechanisms by which it activates caspases remain unknown. To investigate the conservation of CED-4 function in evolution, transgenic *Drosophila* lines that express CED-4 in the compound eye were generated. Ectopic expression of CED-4 in the eyes induced massive apoptotic cell death through caspase activation. An ATP-binding site (P-loop) mutation in CED-4 (K165R) causes a loss of function in its ability to activate *Drosophila* caspase, and an ATPase inhibitor blocks the CED-4-dependent caspase activity in *Drosophila* S2 cells. Immunoprecipitation analysis showed that both CED-4 and CED-4 (K165R) bind directly to *Drosophila* caspase drICE, and the overexpression of CED-4 (K165R) inhibits CED-4-, ecdysone-, or cycloheximide-dependent caspase activation in S2 cells. Furthermore, CED-4 (K165R) partially prevented cell death induced by CED-4 in *Drosophila* compound eyes. Thus, CED-4 function is evolutionarily conserved in *Drosophila*, and the molecular mechanisms by which CED-4 activates caspases might require ATP binding and direct interaction with the caspases.**

In the nematode *Caenorhabditis elegans*, three genes, *ced-3*, *ced-4,* and *ced-9*, play critical roles in the execution of programmed cell death (1–3). In a loss-of-function mutant of *ced-4*, 131 somatic cell deaths that occur during normal nematode development are completely blocked (4). Genetic studies in *C. elegans* have suggested that *ced-4* acts to induce cell death upstream of or parallel to *ced-3*, which encodes a cysteine protease homologous to the mammalian caspase family, and that *ced-9* exhibits its inhibitory effect partly through *ced-4* (5, 6). CED-4 physically interacts with CED-9 and CED-3 (7–10) or with caspase-1, caspase-8, and BCL-X_L (7), which are the mammalian homologues of these *C. elegans* cell-death genes. Many recent studies suggest that CED-4 acts as positive regulator of caspases by enhancing the processing of procaspases to their mature forms. In mammalian 293 cells and insect SF21 cells, the processing of the proform of CED-3 to its mature form is facilitated by CED-4, resulting in the acceleration of CED-3-induced cell death (11, 12). *In vitro* studies by Chinnaiyan *et al.* (13) showed that CED-4 can directly activate CED-3 and that ATP hydrolysis associated with the ATP binding site (P-loop) of CED-4 is required for CED-3 processing. Additionally, Apaf-1, a mammalian homologue of CED-4 that also has a P-loop motif, activates caspase-9 in the presence of cytochrome *c* and dATP, resulting in the sequential activation of caspase-3 *in vitro* (14, 15). More recently, the CED-3/CED-4 complex was shown to be activated by the oligomerization of CED-4 (16). However, the molecular mechanisms for initiating caspase activation remain poorly understood for organisms across the phylogenic scale (11, 12, 17). In contrast to *C. elegans*, multiple caspases are involved in the execution of the cell death program in *Drosophila* (18–20). *Drosophila* contains at least three caspases, DCP-1, drICE, and DCP-2 (21–23), and apoptosis in *Drosophila* can be induced in response to various stimuli, as in mammalian cells (24). Therefore, we expect *Drosophila* to be an appropriate animal model for studying the activation mechanism of the caspase family *in vivo*. In addition to genetic studies, biochemical analyses, and death-inducing activities of cell-death genes can be performed in the *Drosophila* S2 cell line. We have reported that CED-9 inhibits CED-3-induced cell death in *Drosophila* S2 cells (25). This suggests that the molecular components that work through CED-9 prevent CED-3-induced cell death and that these components, including the CED-4 homologue, are conserved in *Drosophila.* A simple hypothesis is that the *Drosophila* homologue of CED-4 can directly activate caspases to execute the cell-death program, and that *C. elegans* CED-4 can mimic its effect in *Drosophila*. In the present study, we show that the function of CED-4 in *Drosophila* is evolutionarily conserved, and we performed further functional and biochemical analyses of CED-4. A P-loop mutation of CED-4 acts as an antiapoptotic molecule and prevents the caspase activation normally induced by CED-4 and by ecdysone. Our results strongly suggest that ATP is required for caspase activation and thus identify a unique molecular mechanism for caspase activation initiated by CED-4 that is evolutionarily conserved.

MATERIALS AND METHODS

Production of Transgenic Flies. The fragment containing the full-length CED-4 or CED-4 (K165R) coding region was ligated into *pGMR* (*G*lass *M*ultimer *R*eporter) (26). The resulting plasmids *pGMRced-4* and *pGMRced-4 (K165R)* were injected into w^{1118}*; Dr/TMS, Sb P*[ry^+, $\Delta 2-3$] embryos as previously described (27). Several independent transformant lines showing similar phenotypes were obtained. *Drosophila* stocks used in this study were *GMR-p35* (26), *GMR-diap1* (28), and *GMR-diap2* (28). *Canton-S* or $white^{1118}$ was used as a wild-type strain.

Histology, Immunohistochemistry, and *in Situ* Caspase Detection. Flies were prepared for scanning electron microscopy as described (29). Semithin sections of adult heads were prepared as described (27). Acridine orange staining to detect dead cells was performed by methods previously described (30).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

This paper was submitted directly (Track II) to the *Proceedings* office.
Abbreviations: *GMR*, glass multimer reporter; FSBA, 5′-p-fluorosulfonylbenzoyl adenosine; IAP, inhibitor of apoptosis protein; DIAP, *Drosophila* homolog of inhibitor of apoptosis protein; RT-PCR, reverse transcription–PCR.

§H.O. and M.M. contributed equally to this work.
¶To whom correspondence should be addressed. e-mail: mmiura@nana.med.osaka-u.ac.jp.

Immunohistochemistry of eye discs was carried out essentially as described (31), except that discs were fixed in 4% paraformaldehyde in PBS. For anti-ELAV staining of third-instar eye discs, primary antibody (7E8A10) purchased from Developmental Studies Hybridoma Bank, Iowa City, was diluted 1:10 in 0.3% Triton X-100 in PBS. Cy3-labeled anti-rat IgG (1:100 dilution, Chemicon) was used as the secondary antibody.

In situ affinity labeling (32) of eye discs from third-instar larvae was performed by using a specific substrate for caspase-3-like protease (PhiPhiLux G6D2, OncoImmunin). Live discs were incubated in RPMI-1640 medium containing 10 μM substrate for 1 h at 37°C in a 5% CO_2 incubator and washed with flow cytometry dilution buffer (OncoImmunin). Samples were mounted and examined with an Axioplan2 fluorescence microscope (Zeiss).

Expression Vectors. cDNAs encoding full-length CED-4, CED-3, and *Drosophia* homolog of inhibitor of apoptosis protein 2 DIAP2 were cloned into p*CaspeR-hs* (33) under the control of the *hsp70* promoter. The coding sequences of *dcp-1*, *drICE,* or *dcp-2* were amplified by reverse transcription–PCR (RT-PCR) from total RNA prepared from 8–18 h *Drosophila* embryos or S2 cells by using the following primers: *dcp-1*, 5′-TTGGATCCATGACCGACGAGTGCGTAACC-3′ and 5′-AAGGATCCCTAGCCAGCCTTATTGCCGTT-3′; *drICE*, 5′-AAGAATTCCATGGACGCCACTAACAATGGA-3′ and 5′-TTGAATTCTCAAACCCGTCCGGCTGGAGC-3′; *dcp-2*, 5′-AAGGATCCATGCTGTTGAAGAGCCTCTACAGG-3′ and 5′-TTGGATCCTCACAGACGAGGTGGAAAGTACAC-3′. Amplified fragments were inserted into p*CaspeR-hs*. The construction of p*CaspeR-hs-lacZ*, *-p35*, *-reaper*, and *-ced-9* was previously described (24).

Cell Culture, Transfections, Cell-Death Assay, and Preparation of Cytoplasmic Lysates for Caspase Assay. S2 cells (34) were cultured, transfected, and used for the cell-death assay as described previously (25, 35). Cytoplasmic extracts of S2 cells were prepared essentially as described (36) with several modifications. At the indicated time after heat-shock treatment, 4×10^2 cells were washed twice with PBS, collected and resuspended in 50 mM Tris (pH7.5)/1 mM EDTA/10 mM EGTA/10 μM digitonin. Cells were incubated at 37°C for 10 min and lysates were clarified by centrifugation at 10,000 $\times$ *g* for 10 min. The cleared lysates containing 5 μg protein were mixed with an equal volume of assay buffer (20 mM Hepes (pH7.4)/100 mM NaCl/0.05% NP40/5 mM $MgCl_2$) and preincubated at 37°C for 30 min (final protein concentration was 0.5 μg/μl). Assay buffer (1 ml) containing 10 μM enzyme substrate Ac-YVAD-MCA or Ac-DEVD-MCA (Peptide Institute, Osaka) was added to the lysate, and the mixture was incubated at 37°C for 30 min. The levels of released 7-amino-4-methylcoumarin were measured by using a spectrofluorometer (Biolumin 960, Molecular Dynamics) with excitation at 380 nm and emission at 460 nm. Activity was expressed as pmol of 7-amino-4-methylcoumarin generated per 30 min per 100 μg of the total extract protein at 37°C. The data were collected from three independent experiments and were represented as means ± SD.

Affinity Labeling of Active Caspase, Immunoprecipitation, and Western Blotting. The affinity labeling was performed by using biotin-DEVD-amk (Biosyn Diagnostics) followed by the procedure described previously (37).

Immunoprecipitation was performed as previously described (38). S2 cells (1×10^2 per 6 cm dish) were transiently transfected with *His_2-tagged drICEmt (C211A)* and/or *ced-4*, then heat shocked. At 24 h after heat shock, cells were harvested, packed by centrifugation, and lysed by 80 μl Nonidet P-40 lysis buffer (38) without DTT. ProBond Resin (50 μl) (Invitrogen) was blocked with control S2 cell lysates for 1 h at 4°C then incubated with cell lysates overnight at 4°C with rotation. The resin was washed five times in Nonidet P-40 lysis buffer. Aliquots of the total lysates and precipitates were separated by 12.5% SDS/PAGE, transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore) and then probed with anti-CED-4 or anti-drICE mouse antisera (described below). These antisera were used for Western blotting at a 1:500 dilution in 4% skim milk/TBST [25 mM Tris (pH 7.5)/150 mM NaCl/0.2% Tween 20]. Horseradish peroxidase-conjugated secondary antibodies (antimouse IgG, EY Laboratories) were used at a 1:500 dilution, and signals were visualized by enhanced chemiluminescence (Amersham).

Antisera were produced by the following procedure. ORFs corresponding to full-length *ced-4*, *dcp-1*, and *drICE* were cloned into p*RSET* (Invitrogen) to allow the expression of His_2-tagged CED-4, DCP-1, and drICE in *Escherichia coli.* These constructs were transformed into BL21, and expression was induced by incubation in a 1,000-ml liquid culture with 1 mM IPTG at 37°C for 4 h. These proteins were separated by SDS/PAGE, the band corresponding to the His_2-tagged protein was excised, and the proteins were electroeluted and used to immunize rats, mice, or rabbits.

RESULTS AND DISCUSSION

GMRced-4 Transgenic Flies Exhibit a "Rough-Eye" Phenotype. To investigate the ability of CED-4 to kill cells in *Drosophila*, we generated transgenic flies that misexpress CED-4 in the *Drosophila* compound eye. The eyes of transgenic flies carrying one copy of the *GMRced-4* transgene showed roughened morphology and reduced size, compared with wild-type eyes (Fig. 1 *A* and *B*). Consistent with this observation, the cellular structures of pigment cells as well as photoreceptor cells were hardly detectable in the *GMRced-4* ommatidia (Fig. 1*E*), in contrast with the wild-type cells (Fig. 1*D*). Although the overexpression of *ced-4* (one copy) was as effective as one copy of *GMRrpr* and *GMRgrim* and was less effective in reducing the size of eyes than was *GMRhid* (18, 39, 40), CED-4 affected both photoreceptor cells and pigment cells, suggesting that the effect of CED-4 on ommatidia development would be broad and would include a variety of cell types.

To analyze the mechanisms underlying excessive cell death in the eyes caused by CED-4 overexpression, we examined the effects of antiapoptotic genes on this process. Baculovirus p35, which inhibits a broad spectrum of caspases (41) and is known to prevent cell death in *Drosophila* (18–20, 26), was coexpressed with *GMRced-4* in the eye. The size of the *GMRced-4* eyes was significantly rescued by the coexpression of p35 (Fig. 1*C*). Sections through the eyes revealed that some of the photoreceptor and pigment cells survived until eclosion (Fig. 1*F*). These results imply that overexpression of CED-4 caused cell death through apoptotic pathways that include the activation of caspases. Overexpression of either DIAP1 or DIAP2, *Drosophila* homologues of the baculovirus inhibitor of apoptosis protein (IAP), have been shown to prevent apoptosis induced by *rpr*, *hid,* and *argos* in the eye (28, 42). Coexpression of DIAP1 or DIAP2 with CED-4 partially prevented the decrease in eye size seen in *GMRced-4* flies, but these inhibitory effects were significantly less than observed with p35 (data not shown).

Ectopic Expression of CED-4 Induces Cell Death in the *Drosophila* Larval Eye Disc with Caspase Activation. The reduction in eye size seen in the *GMRced-4* transgenic flies may have resulted from excess cell death in the eye disc. To examine this possibility, eye discs were stained with acridine orange to detect apoptotic cell death. In eye discs from wild-type third-instar larvae, a small number of dying cells was observed, mainly posterior to the morphogenetic furrow (Fig. 1*G*). In contrast, a greater number of apoptotic cells could be detected behind the furrow in the *GMRced-4* transgenic larvae (Fig. 1*H*). We also examined the differentiation of photoreceptor cells by labeling larval eye discs with anti-ELAV antibody, a nuclear marker for all *Drosophila* neurons. There were fewer ELAV-positive photoreceptor cells and their arrangement was disordered in the posterior region of the *GMRced-4* eye discs, where ectopic cell death occurred (Fig. 1*H*), compared with wild type (compare Fig. 1 *J* and *K*). Coexpression of p35 with CED-4 prevented ectopic cell

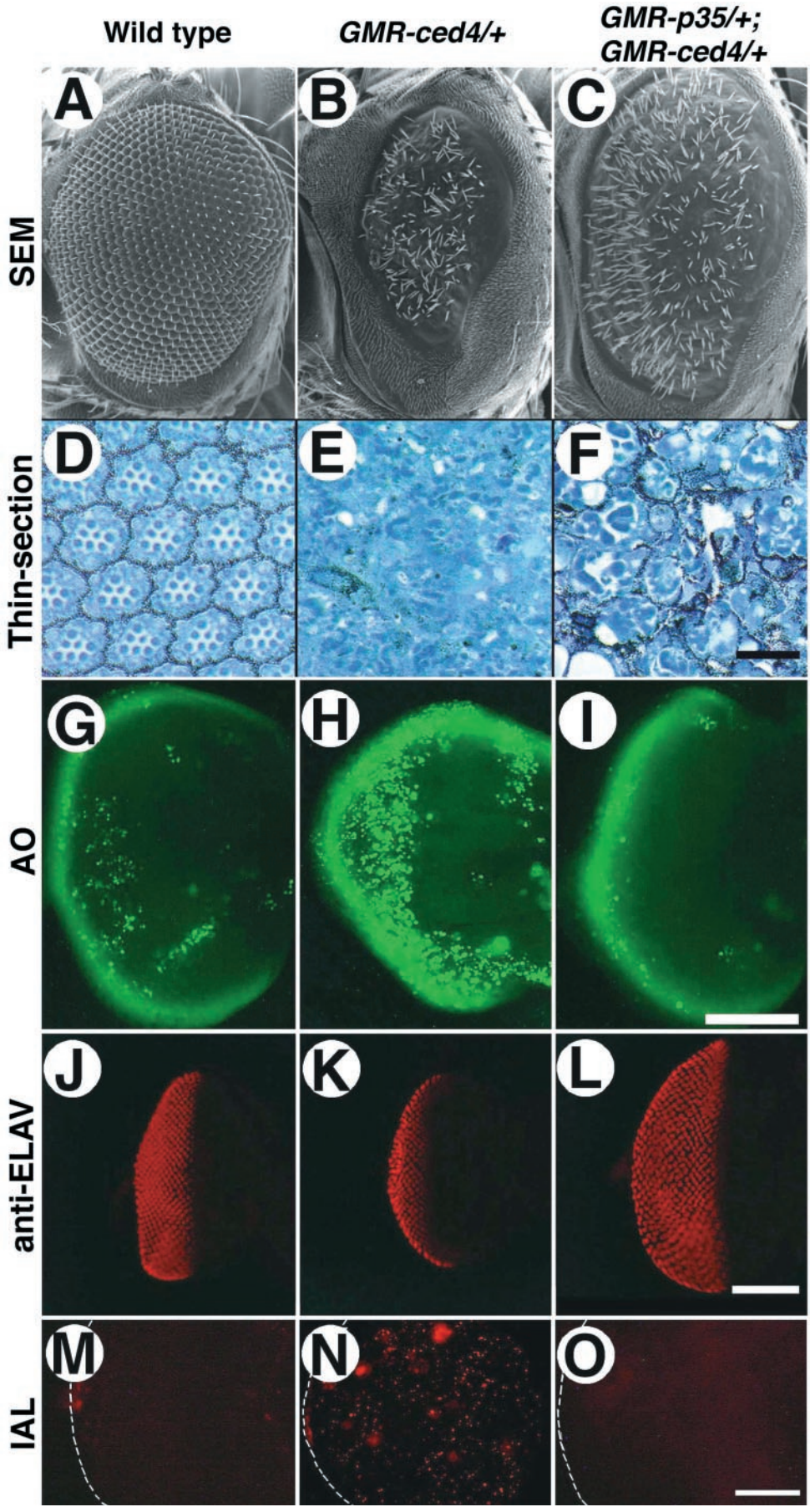

FIG. 1. (*A–F*) The phenotypes of *GMRced-4* transgenic flies are partially blocked by p35**.** (*A* and *D*) Wild-type compound eye. (*B* and *E*) Ectopic expression of one copy of *GMRced-4* causes a reduction of eye size and disordered cell arrangement in the eye. (*C* and *F*) The size of the *GMRced-4*-expressing eye was partially rescued by the coexpression of p35. (*A–C*) Scanning electron microscope images. (*D–F*) Thin sections of adult eyes. Scale bar, 10 μm. (*G–O*) Ectopic expression of *GMRced-4* induces cell death and caspase activation in eye discs from third-instar larvae. In this figure, anterior is to the right and posterior is to the left. (*G, J, M*) Wild-type. (*H, K, M*) Third-instar eye disc from *GMRced-4* larvae. (*I, L, O*) *GMR-p35; GMRced-4*. (*G–I*) Acridine orange staining (AO) to detect dead cells. Scale bar, 100 μm. (*J–L*) Immunostaining by using anti-ELAV antibody. Scale bars, 100 μm. (*M–O*) *In situ* affinity labeling (IAL) of active caspases in third-instar larval eye discs. The dotted line indicates the outer shape of the eye disc. Scale bars, 80 μm. In each experiment, immunohistochemical analysis revealed the expression of CED-4 in larval eye discs, and CED-4-positive cells were distributed in the posterior of the morphogenetic furrow (data not shown).

death and the decrease in ELAV-positive cells (Fig. 1 *I* and *L*), suggesting that the eye phenotype of *GMRced-4* is mostly caused by ectopic apoptosis induced by caspases.

The fact that the phenotypes of CED-4 transgenic flies were blocked by the coexpression of p35 raised the possibility that CED-4 activated the endogenous *Drosophila* caspases in the developing eyes. To investigate this hypothesis, we examined the active forms of caspases in larval eye discs by *in situ* affinity labeling, using a fluorescent substrate for the caspase-3-like protease. In contrast to wild type, large numbers of labeled cells were seen in the CED-4-expressing eye discs (Fig. 1 *M* and *N*). Additionally, coincident with the results from the eye phenotype, the caspase activation detected in the eye discs expressing CED-4 was significantly inhibited by the coexpression of p35 (Fig. 1*O*). These results strongly indicated that CED-4 activated *Drosophila* caspase-3-like caspases, resulting in massive cell death *in vivo* and leading to the eye phenotypes of the *GMRced-4* transgenic flies.

Although the increase of both caspase activity and apoptotic cells in the larval eye discs of CED-4 transgenic flies was completely blocked by the overexpression of p35, the inhibitory effect of p35 on the external morphology of compound eyes was less effective than against the activation of caspases and induction of cell death. To see whether this result could be modified by increasing the dosage of p35, we generated CED-4 transgenic flies that carried two copies of *p35* (*GMR-p35/GMR-p35;GMR-ced-4/+*). Two doses of caspase inhibitor p35, however, were no more effective in preventing the rough-eye phenotype than was one (data not shown). We cannot exclude the possibility that CED-4 induces developmental defects unrelated to apoptosis. Alternatively, it might be possible that CED-4 is also involved in the caspase-independent cell death. CED-4 induces cell death in *Schizosaccharomyces pombe* that is not inhibitable by p35 (16), and Bax and Bak, which are known proapoptotic proteins that function upstream of the caspases, can also induce cell death in a way that cannot be prevented by inhibitor (43, 44).

Overexpression of CED-4 Activates Endogenous Caspases in *Drosophila* S2 Cells. To examine in more detail the role of CED-4 in activating the endogenous apoptotic pathway and inducing cell death in *Drosophila*, we analyzed the effect of CED-4 expression in the *Drosophila* S2 cell line. Transient expression of CED-4 induced cell death with rapid chromatin condensation and approximately 23.7% of the S2 cells underwent apoptosis because of the overexpression of *ced-4* (Fig. 2 *A* and *B*). Next we transfected *ced-4* together with the antiapoptotic genes *C. elegans ced-9*, baculovirus caspase inhibitor *p35*, and *diap2*. In our transient transfection experiments, the overexpression of CED-9 most effectively prevented CED-4-induced cell death, and p35 inhibited apoptosis moderately. Transfection of *diap2* could block *rpr*-induced cell death in S2 cells (data not shown) but not CED-4-induced cell death, indicating that the CED-4-induced cell death pathway does not include the mechanisms that are inhibited by DIAP2. These results suggested that an apoptotic pathway containing certain elements targeted by CED-4 is present in *Drosophila* S2 cells.

The caspase activities were measured in S2 cells expressing CED-4 by using two kinds of specific substrates that distinguish caspase-1 like proteases (Ac-YVAD-MCA) from caspase-3 like proteases Ac-DEVD-MCA. High DEVD- but not YVAD-cleaving activities were observed in the cytoplasmic lysates from *ced-4*-transfected S2 cells (Fig. 2*C*). These activities were effectively inhibited by the coexpression of *p35* or *ced-9* (Fig. 2*D*). In contrast, *diap2* expression could not inhibit the activation of caspases by CED-4 (Fig. 2*D*), consistent with the results of the cell-death assay in S2 cells (Fig. 2*B*). In insect cells (SF21), Op-IAP and Cp-IAP cannot block active-form caspase-dependent cell death (45), and the IAP family also binds to RPR, HID, and GRIM (46), which are proapoptotic proteins known to activate caspases in *Drosophila* (18–20), suggesting that these IAPs act upstream of caspase activation for the inhibition of cell death. These data suggest that CED-4 could enhance the activation of endogenous caspase-3 like proteases in S2 cells, and the inhibitory effect of CED-9 on CED-4-induced caspase activation in S2 cells was consistent with the antiapoptotic function of CED-9 against CED-4 in *C. elegans* (5, 6).

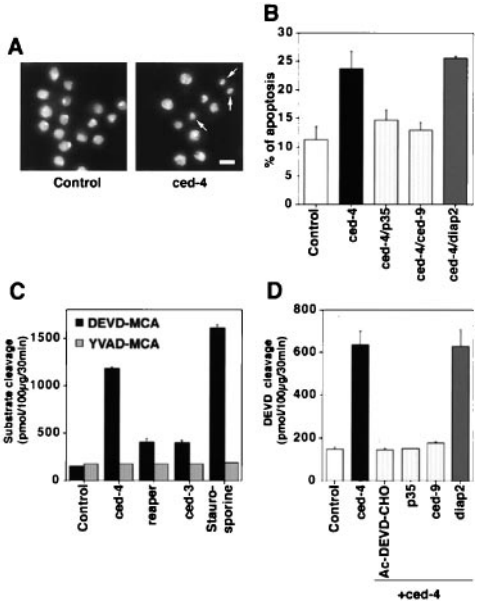

FIG. 2. Overexpression of CED-4 induces cell death and caspase activation in *Drosophila* S2 cells. (*A*) Overexpression of CED-4 kills *Drosophila* S2 cells. Cells were transfected with *ced-4*. At 24 h after heat-shock treatment, cells were fixed and stained with Hoechst 33342. Open arrowhead indicates condensed nuclei. Scale Bar, 10 μm. (*B*) Quantification of cell death in *Drosophila* S2 cells. The data (mean ± SD) are the percentage of cells with condensed chromatin in the total number of cells counted. (*C* and *D*) DEVD- and YVAD- cleaving activities of the cytoplasmic lysates of S2 cells expressing various proteins or treated with 1 μM staurosporine. S2 cells were transfected with various plasmids, and lysates were prepared 24 h after heat-shock treatment.

CED-4 Enhances the Caspase Activities of DCP-1 and drICE. To date, three caspases have been identified in *Drosophila*, DCP-1, drICE, and DCP-2 (21–23). Because *Drosophila* caspase-1 (DCP-1) and drICE can cleave DEVD tetrapeptides as substrates (21, 22, 47), they might be candidates for CED-4-activated caspases. In fact, the DEVD-cleaving activity driven by DCP-1 or drICE was strongly enhanced by CED-4, resulting in the induction of cell death (Fig. 3 *A* and *B*). These data were consistent with previous observations that the autoprocessing of pro-CED-3 is enhanced by CED-4 in 293 cells and SF21 cells (11, 12). We then characterized the CED-4-induced caspases by affinity labeling, which can detect activated caspases by using biotin-DEVD-amk. An active caspase of approximately 24 kDa was detected in the cytoplasmic lysates from *ced-4*- or *drICE*-transfected S2 cells (Fig. 3*C*). Fraser *et al*. (47) have shown that drICE is the essential caspase that executes the cell death program induced by *rpr* in S2 cell lysates. The p24 band induced by CED-4 is identical in size to that of the *rpr*-induced caspase and was detected by immunoblotting by using an anti-drICE antibody (data not shown). RT-PCR analysis revealed that drICE is the most abundant caspase expressed in S2 cells (Fig. 3*D*). Therefore, the CED-4-activated caspase is likely to be drICE in S2 cells. The S2 cell lysates overexpressing DCP-1 contained at least two distinguishable caspases (24 kDa and 22 kDa, Fig. 3*C*). The larger caspase is the same size as *rpr*- or CED-4-induced caspase. The smaller was identical with the large subunit of DCP-1 (p22), as revealed by immunoblotting by using specific antisera against DCP-1 (data not shown). These results further support the idea that CED-4 primarily activates drICE in S2 cells.

CED-4 Requires ATP and Direct Interaction with drICE for Activation. To elucidate the molecular mechanisms underlying

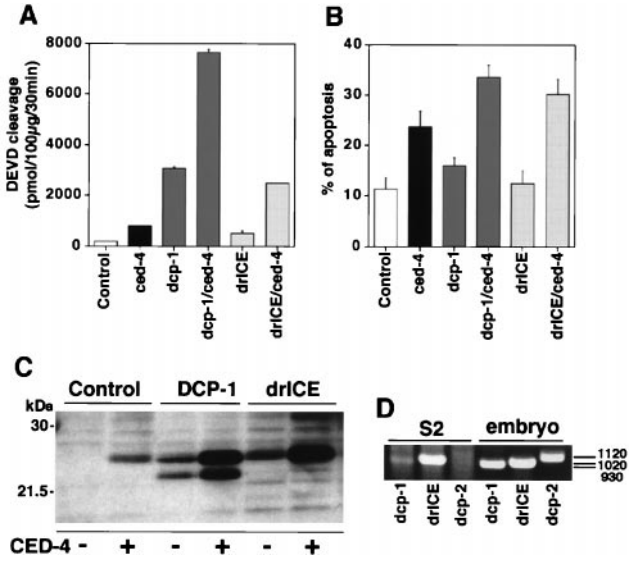

FIG. 3. CED-4 activates DCP-1 and drICE in *Drosophila* S2 cells. (*A*) CED-4 enhanced the caspase activities of DCP-1 and drICE in S2 cells. S2 cells were transfected with *ced-4* and either *drICE* or *dcp-1*, and the caspase activities (mean ± SD) were measured 24 h after heat shock. (*B*) CED-4 promoted cell death induced by *Drosophila* caspases (mean ± SD). S2 cells were transfected with the same combinations shown in (*A*), and the ratio of chromatin condensed cells (% of total cells: mean ± SD) were counted 24 h after heat-shock. (*C*) Activation of caspases as revealed by affinity labeling (see *Materials and Methods*). *ced-4* was cotransfected with either empty vector, *drICE*, or *dcp-1* in S2 cells, and cell lysates for the affinity labeling were prepared 24 h after heat-shock. (*D*) Expression of *Drosophila* caspases in S2 cells and *Drosophila* embryos (0–24 h). RT-PCR was performed by using specific primers for *dcp-1*, *drICE*, and *dcp-2* (see *Materials and Methods*).

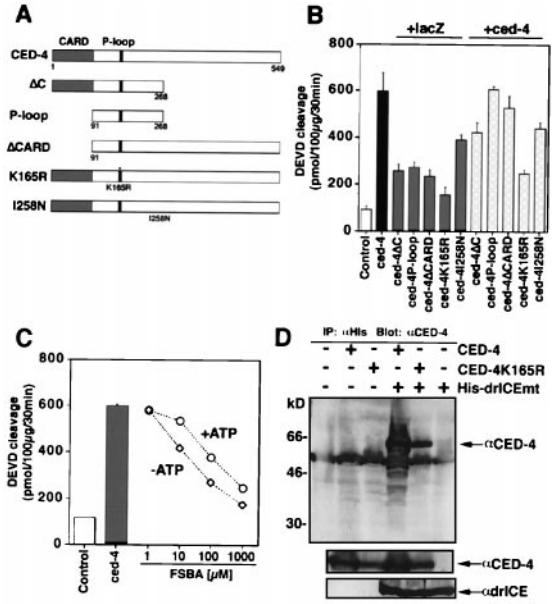

FIG. 4. Direct interaction of CED-4 with drICE and requirement of ATP binding for caspase activation. (*A*) Schematic representation of CED-4 and its mutants. The amino acid sequence in CED-4 is numbered. Single-letter abbreviations for the amino acid residues are as follows; I, Ile; K, Lys; N, Asn; R, Arg. (*B*) CED-4 and its mutants were transfected into S2 cells, and their expressions were confirmed by Western blot (data not shown). CED-4 (K165R) suppressed wild-type CED-4-induced caspase activity in S2 cells. Caspase activities were measured and represented as mean ± SD. (*C*) ATPase inhibitor FSBA blocked CED-4-dependent caspase activity in S2 cells. Cytoplasmic lysates expressing CED-4 were preincubated with various amounts of FSBA, with or without 1 mM ATP at 37°C for 30 min. Caspase activities were then measured (mean ± SD). (*D*) CED-4 and CED-4 (K165R) coimmunoprecipitate with drICE. *His6-tagged drICEmt (C211A)* was cotransfected with *ced-4* or *ced-4 (K165R)* into S2 cells, and cell lysates were prepared 24 h after heat shock, immunoprecipitation, and separation by 12.5% SDS/PAGE (see *Materials and Methods*). Expression of drICE, CED-4, or CED-4 (K165R) in the lysates used for immunoprecipitation experiments is shown (*Lower*).

the activation of drICE by CED-4, we first examined the functional domain of wild-type CED-4 using various mutants (Fig. 4*A*). All the CED-4-mutant constructs we tested exhibited reduced caspase-activating activity (Fig. 4*B*). Activation of DEVD-cleavage activity by CED-4 was reduced by the ATPase inhibitor 5′-p-fluorosulfonylbenzoyl adenosine (FSBA; 48, 49) and by a P-loop mutation, and caspase activity was recovered by the addition of ATP (Fig. 4 *B* and *C*), indicating that binding to and hydrolysis of ATP at the P-loop motif in CED-4 is required for caspase activation. A similar result was reported in CED-4-induced CED-3 activation (13). It is noteworthy that the coexpression of a P-loop mutant, CED-4 (K165R), with the wild-type CED-4 effectively prevented CED-4 from inducing caspase activity (Fig. 4*B*). Subsequently, we examined whether drICE directly interacts with CED-4 in a manner similar to that reported for CED-3 and CED-4, which form a physical complex (7, 10). Immunoprecipitation analysis revealed that overexpressed CED-4 in S2 cells specifically binds to drICE (Fig. 4*D*). Interestingly, CED-4 (K165R) retained its binding activity to drICE, suggesting that CED-4 (K165R) seems to work as a dominant-negative inhibitor for the activation of caspases. These data imply that both a direct interaction between CED-4 and caspases and ATP-binding activity of CED-4 are required for caspase activation.

P-Loop Mutation of CED-4 Converts the Caspase Activator to a Suppresser. We generated a CED-4 (K165R) transgenic fly line (*GMR-ced4K165R*) using an eye-specific promoter. Western blot analysis of eye disc revealed that expression level of CED-4 (K165R) was five times lower than that of CED-4 (data not shown). *GMR-ced4K165R* flies exhibited normal eye morphology (Fig. 5*A*), indicating that the P-loop mutant lost its apoptosis-inducing activity *in vivo*. Interestingly, overexpression of CED-4 (K165R) moderately suppressed the CED-4-dependent reduction of eye size (Fig. 5 *B* and *C*). Because CED-4 (K165R) also suppressed the CED-4-dependent caspase activation in S2 cells (Fig. 4*B*), the restoration of the eye phenotype in CED-4 transgenic flies by coexpression of CED-4 (K165R) is probably because of a decrease in caspase activities. Next, we were concerned about whether the cell-death pathways induced by various stimuli use CED-4 or its homologues for caspase activation in S2 cells. Treatment of S2 cells with cycloheximide (10 μg/ml), staurosporine (1 μM), or ecdysone (20-hydroxyecdysone, 10 μM) for 24 h induced rapid apoptosis and caspase activation (ref. 47 and unpublished data). Ecdysone is an endogenous steroid that regulates metamorphosis in *Drosophila* development and induces apoptosis by means of caspase activation, for example in salivary glands and the midgut, in which apoptotic cell deaths are inhibitable by p35 (50). In our results, caspase activation provoked by cycloheximide and ecdysone in S2 cells were effectively blocked by overexpression of CED-4 (K165R) (Fig. 5*D*). These results suggest that CED-4 homologues of *Drosophila* exist in S2 cells. CED-4 (K165R) may inhibit their function through the heterodimerization, which is thought to be a potent mechanism for caspase activation in CED-4/Apaf-1 (51, 16) or by competing with *Drosophila* CED-4 homologues to bind caspases. In addition, the loss of ATP-binding ability by a P-loop mutation in CED-4 prevents it from activating procaspases; however, ATP binding ability may be not required for CED-4 to bind other components (e.g., drICE). Thus, the P-loop mutant may act as a "dominant negative" molecule.

In conclusion, our results indicate that CED-4 function is conserved in *Drosophila*. Our experiments using the CED-4

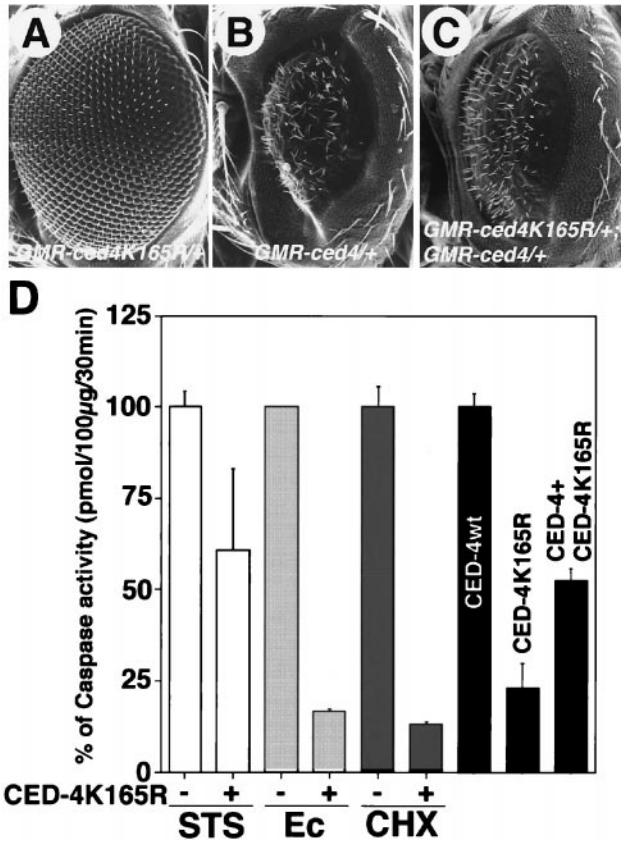

FIG. 5. A P-loop mutation of CED-4 converts the caspase activator to a suppresser. (*A–C*) The phenotypes of *GMRced-4* transgenic flies are moderately inhibited by the ectopic expression of CED-4 (K165R). (*A*) *GMR-ced4 (K165R)/+*. (*B*) *GMR-ced4/+*. (*C*) *GMR-ced4 (K165R)/+;GMR-ced4/+*. (*D*) Overexpression of CED-4 (K165R) suppresses the caspase activation induced by cycloheximide (CHX), staurosporine (STS), and ecdysone (Ec). S2 cells were transfected with empty vector or *ced-4 (K165R)*, then heat shocked, and cycloheximide (10 μg/ml), staurosporine (1 μM), or ecdysone (10 μM) was added. At 24 h after heat shock, the caspase activities were measured (mean ± SD).

(K165R) mutant suggest that a CED-4-like P-loop-containing ATPase is involved in the activation of drICE to promote the cell death program in *Drosophila*. Apaf-1, a mammalian CED-4 homologue, promotes the processing of caspase-3 in the presence of dATP or ATP, cytochrome *c*, and caspase-9 (14, 15). Recent observations showed that ATP is required to execute the apoptotic cell death program (52, 53). Thus, the physical binding of the P-loop-containing ATPase to caspases is a unique mechanism for the activation of caspases, and its role in programmed cell death may be evolutionarily conserved between invertebrates and vertebrates.

We are grateful to Y. Hirota for generously sharing fly head samples, K. Tabuchi for preparing mRNA from fly embryos, Drs. G. Rubin for the fly stocks and *pGMR* vector, J. Yuan for the *ced-4* cDNA, C. B. Thompson for the *dILP/diap2* plasmid, V. M. Dixit for the *p35* plasmid, and Y. Lazebnik for teaching affinity labeling. This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports and Culture to H.O. and M.M., a research grant from Kyowa Hakko Kogyo Co., Ltd., and a Grant-in-Aid from the Tokyo Biochemical Research Foundation to M.M. This work was also supported in part by Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation to H.O.; H.K., and S.H. are research fellows of the Japan Society for the Promotion of Science.

1. Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M. & Horvitz, H. R. (1993) *Cell* **75,** 641–652.
2. Yuan, J. & Horvitz, H. R. (1992) *Development* **116,** 309–320.
3. Hengartner, M. O., Ellis, R. E. & Horvitz, H. R. (1992) *Nature (London)* **356,** 494–499.
4. Ellis, R. E., Yuan, J. Y. & Horvitz, H. R. (1991) *Annu. Rev. Cell Biol.* **7,** 663–698.
5. Shaham, S. & Horvitz, H. R. (1996) *Cell* **86,** 201–208.
6. Shaham, S. & Horvitz, H. R. (1996) *Genes Dev.* **10,** 578–591.
7. Chinnaiyan, A. M., O'Rourke, K., Lane, B. R. & Dixit, V. M. (1997) *Science* **275,** 1122–1126.
8. Spector, M. S., Desnoyers, S., Hoeppner, D. J. & Hengartner, M. O. (1997) *Nature (London)* **385,** 653–656.
9. Wu, D., Wallen, H. D. & Nunez, G. (1997) *Science* **275,** 1126–1129.
10. Irmler, M., Hofmann, K., Vaux, D. & Tschopp, J. (1997) *FEBS Lett.* **406,** 189–190.
11. Wu, D., Wallen, H. D., Inohara, N. & Nunez, G. (1997) *J. Biol. Chem.* **272,** 21449–21454.
12. Seshagiri, S. & Miller, L. K. (1997) *Curr. Biol.* **7,** 455–460.
13. Chinnaiyan, A. M., Chaudhary, D., O'Rourke, K., Koonin, E. V. & Dixit, V. M. (1997) *Nature (London)* **388,** 728–729.
14. Zou, H., Henzel, W. J., Liu, X., Lutschg, A. & Wang, X. (1997) *Cell* **90,** 405–413.
15. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S. & Wang, X. (1997) *Cell* **91,** 479–489.
16. Yang, X., Chang, H. Y. & Baltimore, D. (1998) *Science* **281,** 1355–1357.
17. James, C., Gschmeissner, S., Fraser, A. & Evan, G. I. (1997) *Curr. Biol.* **7,** 246–252.
18. White, K., Tahaoglu, E. & Steller, H. (1996) *Science* **271,** 805–807.
19. Pronk, G. J., Ramer, K., Amiri, P. & Williams, L. T. (1996) *Science* **271,** 808–810.
20. Kondo, T., Yokokura, T. & Nagata, S. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11951–11956.
21. Inohara, N., Koseki, T., Hu, Y., Chen, S. & Nunez, G. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10717–10722.
22. Song, Z., McCall, K. & Steller, H. (1997) *Science* **275,** 536–540.
23. Fraser, A. G. & Evan, G. I. (1997) *EMBO J.* **16,** 2805–2813.
24. Steller, H. (1995) *Science* **267,** 1445–1449.
25. Hisahara, S., Kanuka, H., Yoshikawa, S., Okano, H. & Miura, M. (1998) *J. Cell Sci.* **111,** 667–673.
26. Hay, B. A., Wolff, T. & Rubin, G. M. (1994) *Development* **120,** 2121–2129.
27. Sawamoto, K., Okano, H., Kobayakawa, Y., Hayashi, S., Mikoshiba, K. & Tanimura, T. (1994) *Dev. Biol.* **164,** 267–276.
28. Hay, B. A., Wassarman, D. A. & Rubin, G. M. (1995) *Cell* **83,** 1253–1262.
29. Kimmel, B. E., Heberlein, U. & Rubin, G. M. (1990) *Genes Dev.* **4,** 712–727.
30. Wolff, T. & Ready, D. F. (1991) *Development* **113,** 825–839.
31. Tomlinson, A. & Ready, D. F. (1987) *Dev. Biol.* **123,** 264–275.
32. Clayton, L. K., Ghendler, Y., Mizoguchi, E., Patch, R. J., Ocain, T. D., Orth, K., Bhan, A. K., Dixit, V. M. & Reinherz, E. L. (1997) *EMBO J.* **16,** 2282–2293.
33. Thummel, C. S. & Pirrota, V. (1991) *Drosophila Information Newsletter* **2**.
34. Schneider, I. (1972) *J. Embryol. Exp. Morphol.* **27,** 353–365.
35. Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A. & Yuan, J. (1993) *Cell* **75,** 653–660.
36. Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H. & Tsujimoto, Y. (1996) *Oncogene* **12,** 2251–2257.
37. Faleiro, L., Kobayashi, R., Fearnhead, H. & Lazebnik, Y. (1997) *EMBO J.* **16,** 2271–2281.
38. Vucic, D., Kaiser, W. J., Harvey, A. J. & Miller, L. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 10183–10188.
39. Grether, M. E., Abrams, J. M., Agapite, J., White, K. & Steller, H. (1995) *Genes Dev.* **9,** 1694–1708.
40. Chen, P., Nordstrom, W., Gish, B. & Abrams, J. M. (1996) *Genes Dev.* **10,** 1773–1782.
41. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., *et al.* (1995) *Science* **269,** 1885–1888.
42. Sawamoto, K., Taguchi, A., Hirota, Y., Yamada, C., Jin, M. & Okano, H. (1998) *Cell Death Differ.* **5,** 262–270.
43. McCarthy, N. J., Whyte, M. K. B., Gilbert, C. S. & Evan, G. I. (1997) *J. Cell Biol.* **136,** 215–227.
44. Xiang, J., Chao, D. T. & Korsmeyer, S. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14559–14563.
45. Seshagiri, S. & Miller, L. K. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 13606–13611.
46. Vucic, D., Kaiser, W. J. & Miller, L. K. (1998) *Mol. Cell. Biol.* **18,** 3300–3309.
47. Fraser, A. G., McCarthy, N. J. & Evan, G. I. (1997) *EMBO J.* **16,** 6192–6199.
48. Hartog, A. F., Edel, C. M., Braham, J., Muijsers, A. O. & Berden, J. A. (1997) *Biochim. Biophys. Acta* **1318,** 107–122.
49. van, d. Z., de, G. I., Hartog, A. F. & Berden, J. A. (1997) *Biochim. Biophys. Acta* **1318,** 123–132.
50. Jiang, C., Baehrecke, E. H. & Thummel, C. S. (1997) *Development* **124,** 4673–4683.
51. Srinivasula, S. M., Ahmad, M., Fernandes, A. T. & Alnemri, E. S. (1998) *Mol. Cells* **1,** 949–957.
52. Eguchi, Y., Shimizu, S. & Tsujimoto, Y. (1997) *Cancer Res.* **57,** 1835–1840.
53. Leist, M., Single, B., Castoldi, A. F., Kuhnle, S. & Nicotera, P. (1997) *J. Exp. Med.* **185,** 1481–1486.